## AD NUMBER

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**USAMRMC ltr, dtd 15 May 2003**

AD_____

GRANT NUMBER DAMD17-97-1-7224

TITLE: Characterization of the Breast Cancer Susceptibility Gene, BRCA2

PRINCIPAL INVESTIGATOR: Edward K. Lobenhofer

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 1998 | Annual (16 Aug 97 - 15 Aug 98) |

**4. TITLE AND SUBTITLE**

Characterization of the Breast Cancer Susceptibility Gene

**5. FUNDING NUMBERS**

DAMD17-97-1-7224

**6. AUTHOR(S)**

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Jeff Marks, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Duke University Medical Center
Durham, North Carolina 27710

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Distribution authorized to U.S. Government agencies only (proprietary information, Sep 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

To determine the expression pattern and function of the breast cancer susceptibility gene, BRCA2, we have developed specific immunological reagents against BRCA2. Using these antibodies, we have determined that BRCA2 protein is not detectable in most tissue types, including breast epithelium, suggesting that BRCA2 is present in very low amounts. However, we have demonstrated strong reactivity of our antibodies in differentiating epithelium, indicating that BRCA2 may play a role in cellular differentiation.

Estrogen-induced mitogenesis has been explored at the levels of estrogen receptor mediated transcription and the stimulation of cytoplasmic signaling cascades. Activation of the mitogen activated protein kinase cascade and the phosphatidylinositol 3-kinase pathway are essential for cell cycle progression to occur in response to estrogen. Inhibition of these pathways leads to a cell cycle block early in the G1 stage of the cell cycle between the induction of immediate early gene expression and the increase in cyclin D1 protein levels.

**14. SUBJECT TERMS**

Breast Cancer , BRCA2, estrogen, estrogen receptor, proliferation, differentiation

**15. NUMBER OF PAGES** 30

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Limited |

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Edward K Lobenhofer 9/9/98

PI - Signature Date

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# Introduction

The loss of ability for a cell to accurately control its proliferative activity is one of the defining moments in the development of cancer. The putative mechanisms by which this can occur are numerous and have been the focus of many laboratories. At the time that I wrote the original grant proposal, our laboratory was interested in the breast cancer susceptibility gene, BRCA2. We knew from some of our initial studies that BRCA2 mRNA was cell cycle regulated (1), and by examination of the predicted amino acid sequence for this gene, we found stretches of acidic residues which may serve to function as a transcriptional regulator. The culmination of these facts was the hypothesis that BRCA2 mutations result in transformation due to the abrogation of protein expression of this cell-cycle regulated transcription factor.

Since that time, no data has been gathered implicating BRCA2 as a transcription factor. However, many articles have placed BRCA2's function in the DNA repair cascade. Using a yeast two-hybrid system, Mizuta *et al.* found that murine BRCA2 physically interacts with RAD51, a gene that has been implicated in homologous recombination and DNA repair (2, 3). The increase of chromosomal abnormalities along with a hypersensitive phenotype to gamma irradiation and other genotoxic agents in mouse cells harboring a BRCA2 truncation mutation further define the role of this tumor suppressor gene to DNA repair rather then cell cycle regulation (4, 5). These findings could be extrapolated to humans after the discovery that human RAD51 was shown to interact with the BRC motifs encoded in exon 11 of BRCA2 and that the BRCA2 mutant pancreatic cancer cell line, Capan-1, was hypersensitive to the mutagen methylmethanesulfonate (MMS) (6, 7). The Capan-1 cells could not be rescued by exogenous expression of a BRC-deleted mutant construct; however, wild-type expression of BRCA2 was capable of restoring a normal response to MMS treatment (7).

As is apparent, the evidence for a role of the BRCA2 gene product in recombination repair is mounting. However, there is also a growing body of evidence suggesting the function of this gene in cellular differentiation. In 1996, Rajan *et al.* reported that BRCA2 mRNA is upregulated not only in mitogen stimulated mammary epithelial cells, but also those treated with glucocorticoids resulting in cellular differentiation (8). Another publication suggested that BRCA2 expression was induced in response to puberty, pregnancy and parity, three different stages in which the breast is actively undergoing differentiation as well as proliferation (9). As detailed in the body of this document, the development of molecular reagents for studying BRCA2 function provided us with the necessary tools to gather scientific support for the role of BRCA2 in cellular differentiation.

Due to the pitfalls associated with studying the BRCA2 gene product, which will be delineated in the body of this document, my research has been refocused at addressing the molecular mechanism by which estrogen is capable of stimulating cell cycle progression. Estrogen, a steroid hormone, plays an essential role in female maturation. Occurring simultaneously with the growth of the breasts and ovaries at the onset of puberty is a cyclic pattern of high estradiol ($E_2$) levels, the most active endogenous estrogen. Furthermore, the thickening of the uterine lining during the follicular phase of the menstrual cycle is concurrent with high estrogen levels. These findings suggested that $E_2$ might be involved in cellular mitogenesis. Supporting this claim was the discovery that injection of estrogen into a breast of an animal model lead to the proliferation of the terminal lobular units and a subsequent enlargement of that particular breast (10). Studying immature and ovariectomized rats determined that the mitogenic effects observed in mammary, uterine, and vaginal epithelial cells were caused by estrogen's ability to not only stimulate quiescent cells into the cell cycle, but also to reduce the length of the G1 phase (the stage of cell growth preceding DNA synthesis) (11).

With this proliferative potential of estrogen, the determination that estrogen's cognate receptor, ER, is up to 100-fold overexpressed from basal levels (10 fmols/mg) in 70% of all cases of breast

carcinoma was not surprising (12). These tumors (designated $ER^+$) are generally more differentiated and thus carry a better prognosis than their counterparts, the $ER^-$ tumors. The overexpression of ER appears not to be the result of chromosomal abnormalities resulting in amplification of the gene. Rather, an error in transcriptional regulation has been implicated as evidenced by the elevated levels of AP-2γ, the transcriptional enhancer for ER, and the resulting high levels of ER mRNA (13, 14).

ER is a member of a subset of proteins in the nuclear receptor superfamily that bind hormones, known as the steroid hormone receptors, SHRs. In addition to a DNA binding and a ligand binding domain, SHRs have transcriptional activation functions (AFs). The ER contains two such domains, the first is located in the N-terminal region of the protein, whereas the other (AF-2) is situated near the hormone binding domain. The function of each AF is variable depending upon cell type; however, these domains are synergistic and essential for full ER activity regardless of the cellular context (15-18).

In its unbound state, monomeric ER is bound to a dimer of heat shock proteins (specifically, a Hsp90 homodimer). Hsp90 is theorized to hold the LBD in an unfolded conformation which generates a high-affinity pocket for hormone binding (reviewed by 19). When binding occurs, ER assumes a more structured conformation, which results in the destabilization of the interaction with this heat shock protein dimer. The structural rearrangement of ER is essential for homodimerization and subsequent binding to DNA. This enables interaction of the estrogen bound ER with the basal transcription machinery which then mediates gene expression. Research in yeast expressing recombinant ER determined that Hsp90 is essential for receptor activation (20). This and later studies implicated Hsp90 in the steroid response pathway, potentially through a mitogen activated protein kinase (MAPK) signaling cascade since Hsp90 also complexes with c-src, raf, and MEK, all of which are components of the MAPK pathway (21-23).

A stronger link between estrogen function and the MAPK cascade was made when it was discovered that estrogen simulation of serum-starved cells led to a burst of MAPK activity (24, 25). This estrogen-induced signaling cascade has been found in breast cancer cells, neuroblastoma cells, as well as in bone, uterine smooth muscle, and colon (25-29). The original work on MAPK activation by estrogen suggests that the event is mediated by ER due to the following findings: 1) COS cells expressing recombinant ER activated Erk1 and Erk2, two MAPK family members, 2) this activity was inhibited when treated with a pure anti-estrogen, and 3) untransfected COS cells displayed no response to estrogen treatment (25). Conflicting evidence to this surprising finding (a nuclear receptor mediating cytoplasmic events) has been generated by the demonstration that a plasma membrane-impermeable derivative of estrogen, 17β-estradiol conjugated to bovine serum albumin ($E_2$-BSA), is able to induce MAPK activation, suggesting the involvement of a cell surface molecule in the signaling cascade (29). Although it was discovered that c-*src* is one of the upstream targets of this cascade this did not help clarify the issue since receptor as well as non-receptor mediated mechanisms of MAPK activation can occur through c-*src*'s activation (30, 31).

Shc, p190, and $p21^{ras}$ have also been implicated in the estrogen-induced activation of Erk1 and Erk2 (25). Although, many putative mechanisms exist for activating this signal transduction cascade, the involvement of these specific proteins suggest the following model: c-*src* is activated by estrogen and induces tyrosine phosphorylation of Shc and p190 resulting in the binding of p190 to $p21^{ras}$-GAP. This activation of $p21^{ras}$ turns on the molecular switch for the MAP kinase cascade. Additional work has shown that tyrosine phosphorylation of mitogen-activated protein kinase kinase-1 (MEK-1) in this estrogen stimulated pathway enables this dual specificity kinase to phosphorylate Erk1 and Erk2 (29) These activated MAPKs are then able to traverse the nuclear membrane where they phosphorylate transcription factors, such as Elk1, and induce gene expression of immediate early genes (*e.g.* c-*fos*) (32)

The involvement of c-*fos* in the mitogenic response to estrogen has been well documented, as have the roles of c-*myc* and c-*jun* (reviewed in 33). These immediate early genes, so named for their

ability to be expressed in response to a mitogen without prior protein translation occurring, have all been shown to contain EREs in their untranslated regions (34-36). It is theorized that estrogen bound to ER serves as a transcriptional enhancer for these genes. Immediate early gene expression is only observed for 1 to 2 hours; however, cell division occurs approximately 24 hours later (37). Due to these observations, a model was developed implicating the transcriptional activity following estrogen binding to ER as the mediating event for inducing cellular proliferation.

Two potential mechanisms can now be proposed for the mechanism of estrogen-induced mitogenesis. The first utilizes the ER, as detailed in the previous paragraph, as a transcriptional element to control expression of genes that are essential to stimulate the progression of cells into S phase. The other model states that it is estrogen's ability to stimulate the MAPK cascade, either by an ER-independent or ER-dependent means, that is critical for inducing cellular proliferation. The apparent complexity of estrogen/ER function displays the need for a closer examination of the involvement of estrogen as a signaling molecule as well as a transcription regulator in order to determine the mechanism by which DNA synthesis is stimulated in response to this mitogen.

# Body

**Specific Aims Relating to Work with the BRCA2 Gene and Gene Product.**

In order to study the subcellular localization of the BRCA2 gene product, identify the post-translation modifications the protein undergoes, and explore the potential for protein-protein interactions the need for BRCA2-specific monoclonal antibodies was paramount. To generate GST-fusion constructs to ease and expedite the process of protein purification, a polymerase chain reaction (PCR) was used to amplify two non-overlapping fragments of exon 11, (BRCA2 base pairs (bp) 2716-3714 and 3748-4816, termed BAC1 and BAC2 respectively). Included in the 5' primers was a transcriptional start site (ATG codon) as well as a BamHI restriction enzyme site. The 3' primers were supplemented with a ClaI restriction site. The resulting PCR product was cleaved with BamHI and ClaI and cloned into a Bluescript vector that had been cut with the same enzymes. The resulting "mini-gene" was then subcloned into the pGEX-5X-1 vector (Pharmacia Biotech) in frame with the coding region with the glutathione S-transferase gene from *Shistosoma japonicum*. Both the BAC1-GST and the BAC2-GST constructs were then sequenced to ensure that the PCR amplification had not generated any mutations in the gene. BL21 bacterial cells, which contain a T7 RNA polymerase gene under the control of the IPTG inducible *lacUV5* promoter, were transformed with either the BAC1 or the BAC2 plasmid. Protein purification from IPTG-induced bacteria occurred via two different protocols depending on the desired use of the resulting protein.

BRCA2 peptides to be used in the injection of the mice or rabbits for development of antibodies were purified in the following manner. 100mls of Luria Broth (LB) supplemented with 100μg/ml of ampicillin (amp) was inoculated with either BAC1 or BAC2 transformed BL21 cells. The culture was grown overnight at 37°C. 25mls of this culture was used to inoculate 1L of fresh LB/amp the following morning. Cultures were grown until they reached an $OD_{600}$ reading of 0.4 to 0.75. The culture was chilled on ice for 15 minutes prior to induction with IPTG to a final concentration of 100mM. Induced expression of the BAC fusion protein occurred over a four hour period as the culture was incubated at room temperature with agitation. Cell pellets were generated by centrifugation before being resuspended in an isotonic solution supplemented with protease inhibitors. Cell lysis occurred through the use of lysozyme, freeze-thaw cycles, and sonication as well as the addition of detergent (Triton X-100 at a final concentration of 1%). The soluble fraction of the cell extract was incubated with glutathione sepharose 4B beads to bind the GST portion of the fusion protein. After maximal binding had occurred (overnight incubation at 4°C), the beads were isolated by centrifugation and subjected to extensive washing to remove non-specific proteins. The bound fusion protein was eluted using a solution containing 50mM glutathione and the resulting eluant was dialyzed with "cutting buffer" (150mM NaCl, 50mM TRIS, and 1mM $CaCl_2$). The fusion protein was then cleaved with Factor Xa to separate the BRCA2 peptide from the GST domain. Separation of the two proteins was achieved by running the solution on a 10% SDS-polyacrylamide gel (SDS-PAG) and eluting the 49 kDa band (predicted molecular weight (MW) for the BAC1 and BAC2 portion) by electro-elution.

While this purification scheme resulted in a highly purified BRCA2 peptide without the GST-portion of the fusion construct, the return on investment, in terms of quantity of protein purified, was minimal. For this reason, a "harsher" method was utilized to enable extraction of more BRCA2 peptide from the insoluble fraction of the cell extract. The same methodology detailed above was utilized until a cell pellet was obtained. Cell lysis was performed using a detergent-based (NP-40) lysis buffer followed by sonication. At this point, the soluble fraction of the cell lysate was discarded and the insoluble pellet was subjected to several washes before denaturation using 5M guanidium-HCl. Proteins were then renatured in a glycerol/Tris buffer overnight before being dialyzed with phosphate-buffered saline (PBS). This solution was incubated with glutathione sepharose 4B beads to extract the GST-fusion protein, and the beads were washed extensively at the end of the incubation and subjected to electrophoresis on a 10% SDS-PAG. The protein migrating at the MW of 79 kDa (calculated MW for the BAC fusion protein) was

purified by electro-elution. The resulting GST-BAC fusion proteins were utilized in Western analysis to verify the specificity of putative BRCA2 antibodies.

In colloboration with Lisa Gilliam in Dr. Laura Hale's laboratory, an anti-BRCA2 monoclonal antibody was developed by immunization of mice with the bacterially-produced, gel purified, 49 kDa protein fragment BAC2. Hybridoma formation was performed in the Hale lab as described previously (38).

Validation of monoclonal antibodies and polysera, characterization of the immunoreactivity of the antibodies with terminally differentiating tissue, and determination of the subcellular localization of BRCA2 in these cells was performed in our laboratory and in the Hale lab. The subsequent findings were written up and have been submitted to *Oncogene*. The manuscript can be found in Appendix A of this document. The manuscript marks the completion of not only Specific Aim #1 of my grant proposal, but also a significant portion of Specific Aim #2 (*characterize the BRCA2 protein).*

Specific Aim #3, *to assess the function of BRCA2 by determining the effects of overexpression and downregulation of this gene*, has proven to be a significant challenge. According to observations made in our lab as well as in Dr. Hale's, when a full-length BRCA2 expression construct is transfected into transformed breast epithelial cells cell death occurs. However, it is difficult to ascertain if the effect is due to the expression of the gene or simply the transfection process itself. Further complicating the situation is the size of the BRCA2 gene; it is common practice to transfect cells with genes; however, a gene that has an 11 kilobase (kb) transcript is anything but routine. Additionally, the study of downregulated expression of BRCA2 would be difficult due to the exceedingly low levels of endogenous BRCA2 protein (approximately 50 million MCF-7 cells are needed to detect BRCA2 protein by Immunoprecipitation (IP)-Western analysis using monoclonal antibody 2C9).

Due to these complications, my research efforts have been refocused on studying the molecular mechanisms controlling estrogen-induced mitogenesis. While seemingly a disparate topic, the study of estrogen function is still of significant interest in the field of breast cancer research due the fact that estrogen's cognate receptor, the estrogen receptor (ER), is up to 100-fold overexpressed in 70% of all cases of breast carcinoma (12). As described in the "Introduction" section of this document, the reigning paradigm for estrogen/ER function is that estrogen binds to the ER causing a conformation change of the protein, enabling it not only to dimerize, but also to bind DNA and thus serve as a ligand-dependent transcription factor. Recently, a body of work has been accumulating suggesting that estrogen has the ability to stimulate cytoplasmic signaling cascades; however, the study of estrogen stimulated transcription and the role of these responsive genes in mitogenesis has still remained at the forefront in this field.

**Identification of Estrogen-Responsive Genes Essential in Mitogenesis**

The initial aim of my research regarding estrogen-induced mitogenesis was to identify estrogen-responsive genes that are required for cell cycle progression. Two means for identifying expression differences between estrogen treated and untreated cells have been employed: the PCR-based subtractive hybridization technique known as RDA (described in more detail later) and the use of high density filter arrays.

Gene Discovery Array (GDA) filters contain 18,394 unique human genes and ESTs spotted twice within a 20 cm x 20 cm nitrocellulose membrane. 5μg of poly-A RNA was extracted from MCF-7 cells treated with $2 \times 10^{-8}$ M 17β-estradiol or an equal volume of 100% ethanol (control) and used as template to generate a radiolabelled first-strand cDNA pool through a reverse-transcription reaction. Once the RNA template had been digested to remove the possibility of competitive binding to the clones, the "hot" cDNA was used as a hybridization probe on a filter. After 18 hours of hybridization the filters were

washed extensively. Once washed and exposed to a phosphorimager cassette for 24 hours, potential differences in expression of approximately 20,000 genes was determined. Genome Systems, Inc performed correction for background levels and analysis of clone intensity on individual filters as well as induction differences between the two cell populations.

After screening many of the reportedly "induced genes" by Northern analysis to validate their estrogen-responsive nature, I began to have some serious misgivings about the technique. When the filter screening technique was repeated and the clone intensity assessed, I discovered that my two data sets were not comparable (meaning that the classification of "estrogen inducible" was less than 50% similar between the two rounds of filter screening). This may be a result of Genome Systems, Inc. using bacteria spotted on their membranes rather than cDNA (*ie.* dramatic differences in expression patterns would be expected if the amount of bacteria spotted varied even slightly between filters). On the other hand, the problem may reside in the techniques that I used to develop the radiolabelled probe. However, the fact remains that the use of high-density filter screening did not generate reproducible results in my hands, and for that reason another approach to studying estrogen responsive genes was needed.

To address this issue, a subtractive hybridization protocol, known as representational difference analysis (RDA), was explored. RDA was originally utilized to identify variations between two genomes (39). The technique was then adapted so that expression differences between two cell populations could be readily compared (40). RDA is a three-step process resulting, ultimately, in the cloning of differentially expressed sequences. In the first stage, "representations" of expressed sequences in each cell pool are generated. mRNA is isolated and reverse transcribed to generate a cDNA library for both populations. Each pool is then digested with a restriction enzyme that cuts with a high frequency in order to generate cDNA fragments that will be readily amplified by PCR. Subtractive hybridization ("difference") of the two pools is accomplished by ligating specific primers onto the ends of one population ("Pool A"). By mixing Pool A with a vast excess of the other population (Pool B), representations unique to Pool B will hybridize only to themselves (as is true for species only found in Pool A), and common sequences will form hybrids. Through another PCR amplification: (1) unique representations to Pool B will not be amplified at all; (2) hybrids will only be amplified in a linear fashion, due to the presence of a primer site only on one strand (these species are subsequently degraded using an enzyme that degrades single stranded DNA); and (3) representations found only in Pool A will be amplified exponentially. Repeating this entire process 2-4 times results in a selective amplification of expressed sequences in Pool A represented by discreet bands on an agarose gel. Once the "difference" stage of the protocol is complete, the products are cloned into a PCR product vector and representative clones sequenced and validated by Northerns ("analysis"). For the purposes of this experimentation, I used mRNA extracted from cells treated with estrogen for Pool A and for Pool B the cells were treated with ethanol (vehicle control).

This protocol has the drawbacks of being labor-intensive and the possibility for generating false-positives due to artifacts inherent in the use of *Taq* DNA polymerase. However, the ability to easily standardize the two populations being studied and the potential for identifying novel genes far outweigh the pitfalls. Unfortunately, no promising data has been generated as of yet, but another complete round of RDA will be attempted before the technique is discarded.

**The Role of Cytoplasmic Signaling Cascades in Estrogen-Induced Mitogenesis**

As detailed in the "Introduction" of this document, estrogen has been shown to activate the MAPK cascade. With my revised research focus being on the molecular mechanism of estrogen induced mitogenesis, the potential role of this signaling pathway in the proliferative response to estrogen was of great interest. The first goal was to determine if the MAPK cascade is required for DNA synthesis in response to estrogen. In order to explore this possibility, the cell cycle progression of MCF-7 cells treated with estrogen and the MEK-1 inhibitor, PD098059 was examined. Cells harvested 11.5, 16.5, 19, 20, 24,

and 36 hours after stimulation with estrogen in the presence or absence of 50μM PD098059 were analyzed by flow cytometry following fixation and propidium iodide staining. Progression of cells into the DNA synthesis phase (S phase) of the cell cycle occurred normally in cells treated with estrogen in the absence of this drug. However, prior treatment with PD098059 resulted in complete ablation of cell cycle progression at early time points (*i.e.* within 24 hours after estrogen stimulation). After 24 hours the inhibition was not as complete. However, it is not known whether this effect is due to: 1) the cells overcoming the drug block due to prolonged mitogen stimulation; 2) the drug being degraded either by the media or by cellular metabolism; or 3) the relatively insoluble compound coming out of solution and thus no longer able to directly interact with cellular proteins. Nevertheless, the requirement of MAP kinase activation for progression into S phase of the cell cycle as a result of estrogen stimulation is strongly supported by these findings.

Phosphatidylinositol 3-kinase (PI3-K) activity had been previously shown to be essential for ras and raf activation and subsequent c-*fos* expression in response to estrogen (41). For this reason, LY294002 and wortmannin, inhibitors of the PI3-K cascade, replaced the MEK-1 inhibitor in the protocol detailed above. The data generated from this experimentation suggested that PI3-K activity is also essential for estrogen-induced mitogenesis due to the fact that S-phase values were maintained at or below starvation levels in the presence of these drugs. A broad-based serine-threonine kinase inhibitor, H7, displayed similar results. This was not surprising since phosphorylation of serine 118 and 167 on the ER are essential for its transcriptional activation (42, 43).

The potential for toxicity of these drugs (PD098059, LY 294002, and H7) on MCF-7 cells was ascertained by examining the viability of cells maintained in the presence of the inhibitors for a 24 hour period. In all instances over 95% of all cells were still viable after the 24 hour treatment period, as determined by staining with erythrosin B.

To determine if the inhibitory compounds were having a specific effect on the estrogen response pathway as opposed to being general growth suppressive agents, two components of the p53 growth inhibitory pathway, stabilization of p53 and induction of p21, were studied. Treatment of MCF-7 cells with LY294002, PD098059, and ICI 164,384 (a pure antiestrogen) in the presence of $2 \times 10^{-8}$M estradiol did not result in stabilization of p53 or an induction of p21. This suggested that the inhibitory effects of these drugs on estrogen-induced cell cycle progression is due to specific-block in the mitogenic pathway, rather than a direct activation of an inhibitory pathway. H7 did result in stabilization of p53 and the induction of p21 expression; however, this drug served as a positive control for our experiments since this finding had been previously reported (44, 45).

Immediate early gene expression has been implicated in cellular proliferation and their induction in response to estrogen has been well documented (reviewed in 33). Expression patterns of immediate early genes in response to inhibition of the MAPK and PI3-K cascades were examined in order to determine a mechanism for the cell cycle block. By obtaining total RNA samples from MCF-7 cells treated with estrogen in the presence or absence of PD098059 and LY294002, Northern analysis was utilized to study the expression differences. c-*myc* transcript levels rose approximately five-fold within an hour in response to estrogen in MCF-7 cells and declined to baseline levels by six hours. The same expression pattern was seen in the presence of 50μM PD098059, suggesting c-*myc* induction is not sufficient for mitogenesis. LY294002 did not prevent the induction of c-*myc* expression; however, maximal levels of RNA was delayed approximately 2 hours in response to this drug treatment.

The c-*fos* immediate early gene, an integral portion of the transcription factor AP-1, was then explored for expression in a method analogous to that described above. Induction of c-*fos* levels occurred within one hour of estrogen treatment and basal expression returned within 4 hours in MCF-7 cells. Interestingly, the kinetics of c-*fos* induction in the presence of signaling inhibitors mimicked that which

was observed with c-*myc* expression, PD098059 had no notable effect and LY294002 delayed maximal values by a few hours. Since these inhibitors did not dramatically effect the expression of two essential immediate early genes, we assumed that the cell cycle block induced by these drugs occurred at a later time point.

In order to determine the interval of time in which the blockage occurs, I repeated the cell cycle analysis detailed above with one minor variation. In these experiments instead of pretreating the cells for an hour with the drugs and then stimulating with estrogen, I induced proliferation with estrogen and then at set time points (2, 6, 12, 15, and 18 hours) the drugs were added to the cells. Complete inhibition of cell cycle progression in the presence of LY294002 could still occur if the drug was added 6 hours after induction; however, normal proliferation rates were not observed if the drug was added 15 hours after estrogen treatment. PD098059, which is not as potent of an inhibitor of estrogen-induced mitogenesis, did not have any effect on proliferation rates as long as the drug was added at least 6 hours after estrogen stimulation. Thus, it appeared as if the block was occurring in early G1, between the period of immediate early gene expression and cyclin D1 synthesis.

Expression of cyclin D1 and cyclin E is essential for cell cycle progression into S phase (reviewed in 33). For this reason I examined the expression of these cyclins in estrogen stimulated MCF-7 cells in the presence or absence of PD098059 or LY294002. Cell extracts were prepared at 2, 4, 6, 12, and 24 hours after estrogen treatment and were separated using 12.5% SDS-PAGE. Using commercially available monoclonal antibodies (Santa Cruz) for Western analysis, my results showed that in estrogen stimulated samples cyclin D1 expression was elevated by 4 hours, maximal by 6 hours, and maintained at high levels for the remainder of the 24 hour period. Cyclin E, on the other hand, remained at basal levels until the 24 hour period at which point a two-fold induction of expression occurred. When cells were treated with PD098059 or LY294002 in addition to estrogen, no increase in expression of cyclin D1 or cyclin E was observed.

# Conclusions

The studies involving the BRCA2 gene product have lead us to conclude that, in addition to the function the protein plays in the DNA repair pathway, BRCA2 is also involved in the process of differentiation. Furthermore, the nuclear localization of BRCA2 that had been previously described (46) may be specific to proliferating cells due to our observation that differentiating cells express BRCA2 in the perinuclear region. Our data supports research showing that BRCA2 mRNA is upregulated in differentiating cells (8) and extends these finding to the level of BRCA2 protein expression. Furthermore, our findings are consistent with data which demonstrates that lack of BRCA2 results in abnormalities in differentiating tissues (47).

Induction of the MAPK cascade has been implicated as a consequence of estrogen stimulation of $ER^+$ cells (25). The studies presented in this document not only support this finding, but suggest that the MAPK activation is essential for estrogen-induced mitogenesis to occur. The novel aspect of this finding is that even though estrogen's cognate receptor is located in the nucleus, this steroid hormone is capable of activating a cytoplasmic signaling cascade. Furthermore, our data provides evidence that the function of MAPK activation is required early in the G1 period of the cell cycle, between the expression of immediate early genes and the induction of cyclin D1 synthesis. Interestingly, all of these findings have been extrapolated to the PI3-K pathway.

This report details the importance of signal transduction pathways in estrogen-induced mitogenesis. At this point it is not know if the signaling cascades are being activated by the ER or if estrogen can associate with a cell surface molecule capable of stimulating phosphorylation events. Furthermore, it is yet to be determined if activation of these pathways by estrogen is sufficient to stimulate mitogenesis or if ER-mediated transcriptional activity is also required. Additionally, is the kinase activity associated with these pathways having direct effects on the ER (*i.e.* potentiating the ability for the receptor to dimerization and bind DNA) or other proteins whose function is required for cell cycle progression? However, it is only with additional research focused on the delineation of the molecular mechanism of estrogen induced mitogenesis can we answer these questions and many others that which may lead us to the discovery of targets for potential therapies for the treatment of estrogen responsive tumors.

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# BRCA2 MAbs React with Differentiating Epithelium

By

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## Abstract

Mutations in the BRCA2 gene predispose individuals to early onset, hereditary breast cancer. Although this gene has been extensively studied, very little is known about its protein product or function. In order to better understand the expression pattern and function of the BRCA2 protein, we have developed specific immunological reagents against BRCA2. Using these reagents, we have confirmed that BRCA2 is a high molecular weight protein, consistent with the 384 kDa predicted molecular weight based on its nucleic acid sequence. BRCA2 protein is not detectable in most tissue types, including breast epithelium, except by very sensitive techniques such as immunoprecipitation-Western blot analysis, suggesting that BRCA2 is present in these tissues in very low amounts. However, we have demonstrated strong reactivity of our immunological reagents in differentiating epithelium, including epidermis, thymic epithelium, and squamous cell carcinoma. In addition to BRCA2's putative role in DNA repair, these data suggest that BRCA2 may also play a role in the process of cellular differentiation.

## Introduction

Individuals with germline mutations in the BRCA2 breast cancer susceptibility gene are at increased risk for breast cancer (including male breast cancer), ovarian cancer, pancreatic cancer, prostate cancer, and potentially other malignancies (reviewed in Stratton, 1996). Identified by positional cloning (Wooster et al., 1994; Wooster et al., 1995; Tavtigian et al., 1996), the BRCA2 gene consists of 27 exons extending over 70 kB and encodes a protein with a predicted MW of 384 kDa. The amino acid sequence of BRCA2 shows no significant homologies to other known proteins. Although the BRCA2 gene is generally poorly conserved between species (human BRCA2 is 58% and 59% homologous to rat and mouse, respectively (McAllister et al., 1997)), Bork et al. described 8 copies of a 30-80 aa internally repeated domain within exon 11 (BRC repeats), four of which are highly conserved between rat, mouse, and human (Bork et al., 1996; Bignell et al., 1997; Connor et al., 1997b). Aside from this structural clue, analysis of the BRCA2 gene and its predicted protein product has not contributed significantly to a better understanding of either the normal cellular functions of this protein or how mutation of the gene contributes to development of breast cancer.

The BRCA2 gene is widely transcribed but at relatively low levels in most tissues (Wooster et al., 1995; Tavtigian et al., 1996). Initial tissue screens demonstrated moderate expression of BRCA2 mRNA in normal breast tissue (Tavtigian et al., 1996), with high levels found in thymus and testis, two organs characterized by normally high levels of proliferation and differentiation. High levels of BRCA2 mRNA were seen in murine breast only during development of terminal end buds during puberty and in differentiating alveoli during pregnancy (Rajan et al., 1996). Other organs with significant BRCA2 mRNA expression include ovary, spleen, eye, and certain areas of the brain (Connor et al., 1997b). BRCA2 mRNA expression is regulated by the cell cycle in breast and ovarian epithelial cell lines, with increased BRCA2 mRNA detected in late G1 and early S phases (Rajan et al., 1996; Vaughn et al., 1996). The kinetics of BRCA2 mRNA expression is similar to that observed for BRCA1, leading to the suggestion that these two proteins are regulated in a coordinate fashion (Rajan et al., 1996; Vaughn et al.,

1996). BRCA2 mRNA has been reported to be upregulated in cultured mammary epithelial cells in response to estrogen (Spillman and Bowcock, 1996), as well as in differentiating mammary epithelial cells in response to glucocorticoids (Rajan et al., 1996).

Mice genetically homozygous for BRCA2 truncated prior to exon 11 ("BRCA2-negative") show growth retardation beginning at embryonic day 6.5 and die by embryonic day 8.5 (Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). Post-mortem histologic examination demonstrates lack of appropriate cell numbers and decreased proportion of proliferating cells, leading to the hypothesis that BRCA2 is important in cell proliferation. BRCA2-negative embryos have decreased levels of cyclin E, decreased levels of mdm2, and increased levels of p21 suggesting that BRCA2 protein interacts with the p53 pathway and may similarly function as a "guardian of the genome", to protect the integrity of DNA replication (Suzuki et al., 1997). BRCA2-negative embryonic stem cells are extraordinarily sensitive to radiation exposure which results in (ds) DNA breaks (Sharan et al., 1997). More recent studies have demonstrated that viable mice can be generated using BRCA2 truncated at bp 6038, beyond the BRC repeat regions in exon 11 (Connor et al., 1997a). These mice exhibit growth retardation and die by week 22, primarily of thymic lymphoma. Levels of p21 and p53 are consistently elevated in embryonic fibroblasts homozygous for the truncated BRCA2, however induction of p53 by ionizing radiation appears intact, suggesting that BRCA2 is not involved in sensing dsDNA breaks upstream of p53. Cells homozygous for truncated BRCA2 require increased time to repair dsDNA breaks generated by ionizing radiation, suggesting that BRCA2 is necessary for efficient repair of dsDNA breaks. Studies using the yeast two-hybrid system report that, similar to BRCA1, BRCA2 protein interacts with Rad51, a component of the synaptonemal complexes important in DNA exchange processes during meiosis, and a homologue of the bacterial RecA protein (Shinohara et al., 1992; Mizuta et al., 1997; Scully et al., 1997; Sharan et al., 1997). Furthermore, a recent report has demonstrated that Rad51 binds BRCA2 via the BRC repeat regions, and these domains are required for resistance to methyl methanesulfonate (MMS), which putatively induces double-stranded DNA breaks, in cells lacking full-length BRCA2 (Chen et al., 1998). Taken together, these studies have led to the recent proposal of a "caretaker" role for BRCA2 protein in monitoring and/or repair of DNA double-strand breaks (Kinzler and Vogelstein, 1997; Sharan et al., 1997).

Recently, BRCA2 protein has been immunoprecipitated from nuclear fractions of breast and bladder carcinoma cells, and its expression was shown to be upregulated with the cell cycle, with induction at late G1/early S phase (Bertwistle et al., 1997; Chen et al., 1998). However, no descriptions of the normal cellular and tissue distribution of this protein have been published to date. Here we report the distribution of BRCA2 protein expression using novel monoclonal and polyclonal antisera. We have found reactivity of BRCA2 antibodies with differentiated normal epithelia of skin and thymus, as well as in squamous cell carcinoma of head and neck origin, but not in normal or malignant breast epithelium.

**Results**

*mAb 2C9 Specifically Recognizes BRCA2 Protein.* To determine the specificity of mAb 2C9 (see Materials and Methods), we first performed Western Blot analysis. 2C9 was strongly reactive against 49 kDa purified BAC2 peptide, the immunogen, but was not reactive against the non-overlapping purified BAC1 (Figure 1). In addition, when bacterial lysates containing BAC2 peptide fused to glutathione-S-transferase (GST) were blotted, 2C9 detected a shift in molecular weight to 79 kDa, further confirming the BAC2-specificity of 2C9. 2C9 was not reactive against protein from similar bacterial lysates containing the BAC1-GST fusion protein, or from those producing GST alone (not shown). To further define the BRCA2 epitope recognized by mAb 2C9, we prepared lysates from bacterial or yeast cells transformed with various BRCA2 expression constructs (Figure 2). These studies localized the reactivity of mAb 2C9 to aa 1264-1329, corresponding to bp 4021-4215 of BRCA2 exon 11. In addition, mAb 2C9 reacted with a band consistent with the predicted 384 kDa MW of BRCA2 in Cos-7 cells transfected with full length BRCA2 cDNA (pBSX/BRCA2) by Western blot. The band is identical to that detected by

polyclonal rabbit antiserum #5815, raised against non-overlapping sequences of BRCA2. Negative control mAb P3x63 and normal rabbit serum do not detect bands at this molecular weight (Figure 3).

*BRCA2 is a High Molecular Weight Protein Expressed at Very Low Levels in Breast and Ovarian Carcinoma Cells.* No reactivity above background was demonstrable using mAb 2C9 on frozen sections of breast carcinoma or normal breast tissue by immunohistochemistry, even when using the extremely sensitive immunoenhancing technique of catalyzed signal reporter deposition. Similarly, BRCA2 protein was not detected using 2C9 in standard Western blot assays or flow cytometry of MCF-7 breast carcinoma cells, previously shown to express BRCA2 mRNA under conditions of serum-starvation and release (Vaughn et al., 1996). These studies suggested that BRCA2 protein may be present at very low levels within cells, requiring the use of antigen concentration techniques for detection. Therefore we used 2C9 to immunoprecipitate (and thus concentrate) BRCA2 protein from lysates of breast (MCF-7, T47D, BT483) and ovarian (SKOV3) carcinoma cells, followed by Western blot detection with anti-BRCA2 immunological reagents (polyclonal anti-BRCA2 mouse serum or mAb 2C9). These studies revealed identical high molecular weight bands specifically immunoprecipitated from each breast or ovarian carcinoma cell line, consistent with the predicted size of 384 kDa of the BRCA2 protein (Figure 4).

*BRCA2 mAb 2C9 is Reactive with Terminally Differentiated Normal Epithelial Tissues and with Squamous Cell Carcinomas.* Since mAb 2C9 was non-reactive with normal breast and breast carcinoma tissues, we next screened a panel of acetone-fixed frozen tissues, emphasizing tissues such as thymus, which have high expression of BRCA2 mRNA. The tissues analyzed included skin, thymus, small and large intestine, appendix, liver, kidney, heart, skeletal muscle, tonsil, lymph node, and pancreas. The majority of these tissues are non-reactive with BRCA2 mAb 2C9, as seen for normal breast and breast cancer tissues. However, 2C9 mAb reacted strongly with terminally differentiated thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (Figure 5B), as well as with the suprabasal layers of adult epidermis (Figure 6B). Similarities in expression of a variety of antigens have previously been observed for thymic epithelial cells and epidermal keratinocytes, which is not surprising as both consist of epithelial cells continually undergoing terminal differentiation in the adult (Laster et al., 1986; Patel et al., 1995). Addition of the BAC2 peptide to tissue sections prior to the addition of mAb 2C9 blocked 2C9's reactivity (data not shown). To further determine the specificity of this pattern of reactivity, we then tested monoclonal antibodies directed against different portions of the BRCA2 molecule. MAb 5F6 (raised against a portion of exon 14) also reacts with Hassall's bodies in thymus and with epidermis, although 2 other monoclonal antibodies (3E6 and 9D3) as well as the rabbit polyclonal serum (#5814) were non-reactive (not shown). These differences in reactivity may be due to the availability of the particular epitopes against which each antibody is directed in these tissues, or to differences in Ab reactivity with native protein conformations.

The pattern of reactivity of 2C9 with differentiating epithelium suggested that BRCA2 protein may be stabilized or upregulated under conditions favoring differentiation of epithelium. Therefore, we next examined the reactivity of this antibody with a panel of moderate to well differentiated squamous cell carcinomas (SCCs) of head or neck origin. MAb 2C9 demonstrated strong reactivity with 9 of 10 formalin-fixed paraffin-embedded well differentiated SCCs (Figure 7B). 2C9 was also reactive with 1 of 4 frozen, moderately differentiated SCCs. The frozen tumor which was reactive with mAb 2C9 also reacted with mAb 5F6, directed against a different portion of the BRCA2 molecule (data not shown), suggesting that these antibodies are specifically reacting with BRCA2 in SCCs.

*BRCA2 mAb 2C9 Immunoreactivity Increases in Differentiating Keratinocytes.* We further confirmed mAb 2C9 reactivity with differentiating epithelium using immunoperoxidase assays on epidermal keratinocytes cultured in situ onto glass slides. Keratinocytes cultured in media containing either increased calcium concentrations (1.2 mM) or fetal calf serum (FCS) undergo characteristic changes associated with differentiation, including changes to squamous morphology and upregulation of

molecules that are considered classical markers of differentiation (high molecular weight cytokeratins 1, 2, and 10, filaggrin, loricrin, and transglutaminase). Little 2C9 immunoreactivity is seen in epidermal keratinocytes at baseline (t=0), which is limited to a few scattered cells which are larger, more spread out (squamous), and more differentiated-appearing than cells which are non-reactive with 2C9. Increased 2C9 immunoreactivity is noted after several hours of incubation in differentiation medium, and 2C9 immunostaining remains strongest in the large, squamoid, more differentiated cells (which are much more numerous after several hours of differentiation). The staining pattern in these cells is cytoplasmic with perinuclear accentuation and nuclear dots (Figure 8).

Because the immunohistochemical reactivity of 2C9 correlates with terminal differentiation of epithelial cells, we examined BRCA2 mRNA levels in terminally differentiating keratinocytes by Northern blot analysis. BRCA2 mRNA levels do not appear to change during differentiation of keratinocytes, suggesting that the upregulation of protein in these cells is occurring at a post-transcriptional level (data not shown).

**Discussion**

This study demonstrates that BRCA2 mAbs react with epithelial cells undergoing terminal differentiation, including skin, cultured epidermal keratinocytes, Hassall's bodies in the thymus, and squamous carcinomas. We did not see reactivity with either normal or malignant breast epithelium, suggesting that BRCA2 protein is expressed in these tissues at levels which are undetectable in immunohistochemical analyses. The role of BRCA2 in nuclear activities, primarily DNA damage repair, has been under intensive investigation; however, the involvement of BRCA2 in differentiation is poorly understood. A significant amount of evidence supports the concept that BRCA2 is important in the process of differentiation, in addition to its role in mitogenesis. Initial in vitro studies demonstrated that BRCA2 mRNA expression is cell cycle regulated and decreases to negligible levels during serum starvation or at confluency, when cells are no longer proliferating (Rajan et al., 1996; Vaughn et al., 1996). Rajan et al. further demonstrated that BRCA2 is upregulated in response to lactogenic hormones in post-confluent, non-proliferative cells during mammary epithelial cell differentiation (Rajan et al., 1996). In situ hybridization studies in the mouse have demonstrated that BRCA2 is expressed in many tissues in which cellular compartments are undergoing rapid proliferation, including ovary, testis, lymph node, spleen, thymus, pancreas, endometrium, stomach mucosa, intestinal crypts, epithelium in the outer root sheath of hair follicles, and mammary gland, where expression is upregulated during pregnancy, again suggesting that this protein is important in normal control of the cell cycle (Rajan et al., 1997; Connor et al., 1997b; Blackshear et al., 1998). However, Blackshear et al. also demonstrated BRCA2 mRNA expression in some non-proliferating cell types, including terminally differentiated neurons and more differentiated cells undergoing spermatogenesis (Blackshear et al., 1998). These studies suggest some function for BRCA2 in differentiation processes, unrelated to its involvement in the cell cycle. Further evidence for the role of BRCA2 in the process of differentiation is provided by the phenotype of the mice homozygous for truncated BRCA2 which survive embryogenesis (Connor et al., 1997a), in which some tissues show evidence of improper differentiation, including skin, testes, ovaries, and thymocytes.

Clearly, BRCA2 has important roles in both cellular proliferation and differentiation; however, the mechanisms involved are still unclear. It is difficult to understand how BRCA2's "proliferative" role, as seen in the non-viable or growth retarded BRCA2-deficient mice, might be related to tumorigenesis in humans. Based on the finding that levels of the cyclin-dependent kinase inhibitor p21Cip1/WAF1 are increased in BRCA2 mutant embryos, Suzuki et al. suggested that lack of this protein in BRCA2-deficient mice results in DNA repair defects during the highly proliferative stages of embryogenesis that then activate pathways involved in checkpoint control, culminating in cell cycle arrest (Suzuki et al., 1997). Connor et al. also found that p21, as well as p53, are overexpressed in mouse embryonic fibroblasts derived from embryos homozygous for truncated BRCA2 (Connor et al., 1997a). Furthermore, BRCA2/p53 nullizygotes have a less severe phenotype than mice with BRCA2-deficiency alone (with

average survival time in utero increased from 8.5 days to 10.5 days), suggesting that the upregulation of p53 noted in the BRCA2-null mice, or some downstream effect of this upregulation, is partly responsible for the earlier embryonic lethality in these animals (Ludwig et al., 1997). A model for human tumorigenesis due to loss of BRCA2 has been proposed in which both the loss of the wild-type allele in BRCA2 mutation carriers, as well as loss of the p53 pathway, are required for tumorigenesis (Connor et al., 1997a; Bertwistle and Ashworth, 1998). This model requires that a mammary epithelial cell suffer three "hits" prior to malignant transformation (two hits to knock out the p53 pathway, and one hit to knock out the wild-type BRCA2 allele.) Furthermore, it is likely that the p53 pathway would have to be eliminated first, followed by loss of the wild-type BRCA2 allele, as mutations occurring in the opposite order would probably have the same effect as is seen in BRCA2-null mice, namely growth arrest rather than uncontrolled proliferation (Bertwistle and Ashworth, 1998).

Another possibility to consider in trying to understand BRCA2's contribution to tumor suppression is its putative role in the process of differentiation. Most normal cells in an adult are non-proliferative, unless tissue damage or response to particular hormones or mitogens stimulates these cells to re-enter the cell cycle. Furthermore, most cells that are actively proliferating, aside from the pure population of stem cells, are programmed to exit the cell cycle at some point and enter the differentiation pathway. Mammary epithelial cells would be included in the latter category, in that a subpopulation of these cells is hormonally stimulated to develop into differentiated structures in the breast with each menstrual cycle. Thus, unchecked proliferation in these cells may be consequent to either unresponsiveness to signals that control cell cycle activity, or alterations in a signal that would normally instruct these cells to leave the cell cycle and enter the differentiation pathway. A recent report demonstrated that p21, which has traditionally been considered an inducer of differentiation, is initially upregulated in cultured mouse keratinoctyes following a differentiation signal, but that the protein levels must quickly be downregulated in order for differentiation to proceed (Di Cunto et al., 1998). These data are consistent with a potential role for BRCA2 in a negative feedback mechanism required in differentiating epithelium to eliminate upregulated p21 protein and allow differentiation to proceed normally. This may provide an explanation for the abnormal differentiation of skin and other tissues noted in the mice expressing truncated BRCA2, in which p21 levels are elevated (Connor et al., 1997a). According to this hypothesis, loss of BRCA2 resulting in p21-mediated resistance to normal differentiation processes may be a mechanism for tumorigenesis. Whether p21 is functionally involved in the BRCA2 -/- mutant phenotype (and by correlation in tumorigenesis in humans); and if so, whether the effect of its upregulated expression is due to an anti-proliferative function or to an inhibition of normal differentiation processes has yet to be elucidated.

Bertwistle et al. recently identified BRCA2 protein by immunoprecipitation using multiple polyclonal antisera directed against different portions of the BRCA2 molecule, and detection with a rat monoclonal antibody raised against the C-terminus (aa#3386-3400) of BRCA2. A single high molecular weight species was detected in each case by the rat monoclonal antibody (Bertwistle et al., 1997). Similarly, Chen et al. described a high molecular weight species immunoprecipitated from 35S-labeled T24 bladder carcinoma cells that was consistent with BRCA2, using each of two BRCA2 polyclonal antisera (Chen et al., 1998). In cell lines transfected with the full length BRCA2 construct, we detect a single high molecular weight band consistent with the predicted 384 kDa using our monoclonal and polyclonal antibodies. However, we also see lower molecular weight species in addition to the high molecular weight band in immunoprecipitation-Western blot experiments from breast cancer cell lines. Smaller molecular weight species may derive from alternative splicing events, differential RNA processing, BRCA2 protein processing, protein degradation, or cross-reactivity. Alternatively spliced mRNA species have not been reported for BRCA2, and we see single bands on Northern blot analysis, even in cases in which smaller protein sizes are detected, making alternative RNA splicing a less likely explanation. However, it is possible that our antibodies detect post-translationally processed BRCA2 species which, if

these species did not include the C-terminal portion of BRCA2, would not be detected by the rat monoclonal antibody used in the Bertwistle report.

In both the Bertwistle and Chen reports, these investigators localized BRCA2 protein to the nucleus of carcinoma cells by differential purification of subcellular fractions (Bertwistle et al., 1997; Chen et al., 1998) This protein's interaction with RAD51 and its putative role in double stranded DNA repair are consistent with its localization to the nucleus. However, our experiments have consistently demonstrated BRCA2 mAb 2C9 reactivity in the cytoplasm of differentiating cells, in a perinuclear pattern with nuclear dots. Our results may not be incompatible with studies demonstrating nuclear localization of BRCA2 in carcinoma cells, as this molecule may have different functions, and thus different localization in different cell types or stages of development. Its function as a molecule involved in the process of differentiation may require its presence in the cytoplasm, whereas its function as a DNA repair molecule, or molecule otherwise involved in cell cycle processes, may require its presence in the nucleus. As BRCA1 and BRCA2 appear to be coordinately regulated, and mRNA for both genes appears to have a very similar distribution, it is likely that the protein expression pattern is also similar. A similar ongoing debate over the localization of the BRCA1 protein remains unresolved, with reports of nuclear staining as well as differential trafficking with localization to the cytoplasm in breast cancer cells (reviewed in (Bertwistle and Ashworth, 1998). A more recent report suggested that the "nuclear dot" staining pattern of BRCA1 mAbs was actually due to reactivity located in tube-like channels extending into the nucleus, likely originating from the perinuclear endoplasmic reticulum-Golgi complex (Coene et al., 1997). Of note, the authors report that the nuclear tubes are seen in a fraction of the cell population, that they form a much more extensive branching network in cancer cells compared with non-malignant cells, and that the nuclear invaginations are often seen in close proximity to nucleoli, suggesting a possible involvement in the cell cycle. Coene et al.'s description of a perinuclear staining pattern with nuclear dots for BRCA1, with distinct visualization of either nuclear staining or cytoplasmic staining being dependent on the fixation method, is consistent with the immunohistochemical staining pattern we observe with mAbs against BRCA2. The subcellular localization of BRCA2 in different cell types deserves further investigation.

In our studies, 2C9 clearly reacts immunohistochemically with a cytoplasmic protein in epidermis, cultured keratinocytes, and thymic epithelium. The possibility that the observed 2C9 reactivity in these cells is entirely non-specific, due to cross reactivity with another protein present in skin, thymus, and differentiating keratinocytes, is very unlikely, as mAb 5F6, which is directed against an entirely separate part of the BRCA2 molecule, reacts with these same cells by immunohistochemistry. In addition, 2C9, 5F6, and 3E6 react with identical high molecular weight bands on Western blot from skin extracts, and 2C9 and polyclonal anti-BRCA2 rabbit serum #5815 react with identical bands from cultured keratinocytes undergoing differentiation (data not shown). Although BLAST searching revealed no significant homology to other differentiation-associated proteins at the nucleic acid level, the 65 aa portion of BRCA2 to which the 2C9 epitope has been narrowed demonstrated limited amino acid homology with the cytokeratin 2 (CK2) molecule (45% identity, with 76% similarity; longest stretch of identical aa = 3) over a 22 amino acid stretch (aa #1305-1327). However, our data clearly demonstrates lack of 2C9 cross reactivity with cytokeratins in Western blots, as well as a different immunohistochemical staining pattern compared with that of mAbs recognizing CK2 (Figure 6). Furthermore, CK2 is not found in foreskin epithelium, squamous carcinomas, or their derivative cell lines (Collin et al., 1992), and cytokeratin staining patterns are exclusively cytoplasmic, with no nuclear reactivity. Thus, while it is impossible to totally exclude immunohistochemical cross-reactivity of 2C9 with CK2 in tissue sections, amassed evidence suggests that 2C9 is specifically detecting BRCA2 present in the cytoplasm of differentiating epithelial cells.

In conclusion, direct study of BRCA2 distribution and function within cells has been limited by the lack of appropriate reagents, as well as the fact that the BRCA2 protein is very large and appears to be

expressed at very low levels in most tissues. Utilizing monoclonal antibody 2C9 and polyclonal antibody #5815, developed in our lab, as well as other monoclonal antibodies raised against BRCA2 sequences to confirm specificity, we have demonstrated a potential role for BRCA2 in cellular differentiation of keratinoctyes and thymic epithelium. Our data supports research showing that BRCA2 mRNA is upregulated in differentiating cells (Rajan et al., 1996) and extends these findings to the level of BRCA2 protein expression. Furthermore, our findings are consistent with data which demonstrates that lack of BRCA2 results in abnormalities in differentiating tissues (Connor et al., 1997a). Whether BRCA2 actually plays a role in the process of differentiation in these tissues, or whether this protein's presence in differentiating tissues is simply a result of accumulation due to extensive crosslinking, enhanced stability, or some other factor, has yet to be elucidated. However, BRCA2 likely plays multiple roles in the diverse processes of cellular proliferation, differentiation, and monitoring of the integrity of DNA.

**Materials and Methods**

Antibodies: The anti-BRCA2 monoclonal antibody 2C9 was developed by immunization with a bacterially-produced gel purified 46 kD protein fragment derived from BRCA2 exon 11, termed BAC2 (bp 3748 to 4817, Genbank U43746), and hybridoma formation as described previously (Scearce and Eisenbarth, 1983). Anti-BRCA2 polyclonal rabbit antiserum #5814 was generated by immunization with a bacterially produced peptide derived from a non-overlapping portion termed BAC1 (bp 2716-3714) of BRCA2 exon 11. Additional anti-BRCA2 mAbs generated against sequences in exon 14 (mAbs 5F6 and 9D3) or against a peptide fragment encoded by bp 792-1917, corresponding to mid exon 7 through most of exon 10 (mAb 3E6) were kind gifts of Wen Hwa Lee. Anti-cytokeratin (CK) mAbs AE1 and AE3 (reactive with CK 1-2, 5, 6, 14 ) were obtained from Boehringer Mannheim. Anti-CK mAb AE2 (reactive with CK 1, 2, and 10 found in terminally differentiated cells) was a kind gift from Barton Haynes. Mib-1 mAb specific for the Ki-67 nuclear antigen expressed by all cells that are not in G0 (Key et al., 1993) was obtained from Immunotech Inc. Anti-phosphotyrosine mAb 2G8.D6 hybridoma cell line was obtained from the ATCC and was grown in IMDM+10% Fetal Clone I (Hyclone, Logan UT).

Tissue Culture. MCF-7, BT483, and T47D breast carcinoma cell lines and the SKOV3 ovarian carcinoma cell line, obtained from the ATCC, were grown in RPMI 1640+10% fetal calf serum (FCS). Cos-7 cells were obtained from the Duke University Tissue Culture Facility and were grown in IMDM + 5% FCS. Human foreskin keratinocytes (HFKs) were derived from discarded neonatal foreskins by trypsinization (0.25%, 4°C, overnight) and mechanical dissociation, and were grown in Keratinocyte Serum-free Medium (Life Technologies, Grand Island NY). All cultured cells were grown at 37oC in 5% CO2.

BRCA2 cDNA and Transfectants. Full length BRCA2 cDNA was prepared as follows: a cDNA containing BRCA2 bp 23-10502, encoding full length BRCA2 protein (translated from bp 229 to bp 10485) was created by splicing partial cDNAs derived from an MCF-7 breast carcinoma cDNA library and PCR cloning (bp 9784 to 10502). This DNA was cloned into the pBSX/CMV/pA expression vector, a modified Bluescript under the control of the CMV immediate-early promoter/enhancer and stabilized by SV40 polyA sequences, a kind gift of A. Amalfitano. 10 μg of this construct pBSX/BRCA2 was used to transfect Cos-7 cells, using the Fugene (Boehringer Mannheim, Indianapolis IN) transfection agent according to the manufacturer's protocol. Transfected cells were analyzed after 48 hours in culture by protein extraction and Western Blot (described below).

Immunohistochemistry. 5 mm frozen tissue sections were acetone-fixed and blocked with goat serum for 20 minutes at 37°C. The sections were reacted with primary antibody for 30 minutes at 37°C, followed by a 10 minute wash (10 mM NaPO4, pH 7.5, 0.9% PBS). The bound antibodies were then reacted with biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame CA) for 30 minutes at 37°C, followed by a wash as described above. Finally, the sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame CA) for 30 min at 37°C, washed, and then incubated with 3,3'-

diaminobenzidine(DAB)/H2O2 substrate solution for 5 minutes. Sections were washed, counterstained with hematoxylin, and permanently mounted. To enhance sensitivity in some experiments, signals were amplified using the Tyramide Signal Amplification (TSA) system (NENTM Life Sciences Products, Boston MA) according to the manufacturers instructions. Where indicated, formalin-fixed paraffin-embedded tissue sections were deparaffinized, then heated in Glyca antigen retrieval solution (BioGenex, San Ramon CA) for 2C9 or 0.01M citrate solution for cytokeratins, 2 x 5 minutes at 600 Watts using a microwave oven, prior to the blocking step and further immunostaining as described above.

Western Blot Analysis. Protein extracts were prepared using 10 mM Tris (pH 8.0), 150 mM NaCl, and 1% Triton X-100 in the presence of proteinase inhibitors (20 mM iodoacetamide, 0.02 mM E64, 0.03 mg/ml aprotinin, 0.07 mM pepstatin A, 1 mg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) at a concentration of 50 ml per 10x106 cells. Following a 20 minute incubation on ice, samples were centrifuged for 30 minutes at 14,000 rpm, 4°C to remove insoluble material. Detergent soluble proteins from $5x10^6$ cells were boiled in sample buffer for 5-10 minutes, separated on a 4% or 5% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to nitrocellulose. Blots were then incubated with anti-BRCA2 or control antibodies, followed by HRP-conjugated goat anti-mouse or rabbit IgG, with intervening washes. Antibody-bound protein was visualized by exposure to a chemiluminescent substrate (ECL Western blotting protocol; Amersham Life Science, Arlington Heights, IL) and detection on film.

Immunoprecipitation. Lysates prepared as described above were precleared twice with 20 ml of control P3x63 supernatant, followed by 20 ml of goat anti-mouse IgG-agarose (Sigma, St. Louis MO). BRCA2 protein was then immunoprecipitated with 20 ml of 2C9 supernatant, followed by 20 ml of goat anti-mouse IgG-agarose. For all reactions with primary and secondary antibodies (except 2C9), lysates were incubated for one hour at 4°C with end-over-end mixing; lysates were incubated with 2C9 antibody overnight at 4°C. Following immunoprecipitation, the agarose pellets were washed sequentially with 500 ml of the following solutions: 1) TSA (0.01 M Tris, pH 8, 0.14 M NaCl, 0.025% NaN3) + 0.1% Triton X-100, 2) TSA + 0.1% Triton X-100, 3) TSA, and 4) 0.05 M Tris-Cl, pH 6.8. The pellets were then boiled in sample buffer for 5-10 minutes, the agarose was pelleted, and the supernatant was analyzed by Western Blot as described above.

**Acknowledgments**
The authors would like to thank Drs. Barton F. Haynes, Salvatore Pizzo, and George Cianciolo for helpful discussions and Jie Li, Tamajah Gibson, and Gudrun Huper for expert technical assistance. We would also like to thank Dr. Barton F. Haynes of the Duke University Specialized Center of Research in Rheumatoid Arthritis for assistance with preparation of mAb 2C9. This work was supported by the Duke University Specialized Program of Research Excellence (SPORE) in Breast Cancer (CA68438), and by the Department of Defense Breast Cancer Research Program (DAMD #17-96-1-6016, #17-94J-4168, and #17-97-1-7224).

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**Figure Legends**

Figure 1: Mab 2C9 reacts with immunizing peptide BAC2 and its fusion protein BAC2-GST. Lysates were prepared from uninduced (lane 2) and induced (lane 3) bacteria producing the BAC2-GST fusion protein (BRCA2 bp 3748-4816) as well as from uninduced (lane 4) and induced (lane 5) bacteria producing the BAC1-GST fusion protein (BRCA2 bp 2716-3714). The lysates and GST-cleaved and purified BAC2 protein were separated on a 7.5% polyacrylamide gel. 2C9 was reactive with the purified BAC2 protein (lane 1) at the expected MW of 49 kDa as well as with the 79 kDa BAC2-GST fusion protein (lanes 2, 3). 2C9 was non-reactive with all components of the lysates from BAC1-GST producing bacteria (lanes 4, 5).

Figure 2: Reactivity of mAb 2C9 with BRCA2 Exon 11 Constructs. A) The BRCA2 peptides studied are shown on this diagram of exon 11, along with structural features of this region of the BRCA2 gene, including BRC-repeats #1-8 (Bork et al., 1996) and a region which is highly conserved among different species (Bignell et al., 1997). C) Induced lysates from bacteria or yeast transformed with the indicated BRCA2 sequences were analyzed on 10% SDS-PAGE gels, transferred to nitrocellulose, and reacted with 2C9 in a Western blot assay. 2C9 was reactive with bacterial proteins containing the immunizing peptide BAC2, bp 3748-4816 (lane 1); deletion constructs BAC2-1, bp 3748-4454 (lane 2); BAC2-3, bp 3748-4215 (lane 3); BAC2-4, bp 3987-4215 (lane 4); and yeast-derived BRCA2 sequences, bp 4021-5352 (not shown); but not with deletion construct BAC2-2, bp 3748-3987 (lane 5) or BAC1, bp 2716-3714 (Figure 1), derived from a different portion of BRCA2 exon 11. These studies define the 2C9 epitope to be contained within BRCA2 aa 1264-1329, encoded by bp 4021-4215. Reactivity of BRCA2 deletion constructs with 2C9 is summarized in panel B.

Figure 3: BRCA2 antibodies recognize full length BRCA2 in Cos-7 cells. Lysates from Cos-7 cells transfected with pBSX/BRCA2 (lane 1) or mock transfected (lane 2) were reacted with mAb 2C9 (left panel) or polyclonal rabbit serum #5815 (right panel) in Western blot assays. Identical bands consistent with the 384 kDa predicted molecular weight of BRCA2 were detected by both 2C9 and #5815. Extremely faint bands at the same molecular weight are also seen in lysates from mock transfected Cos-7 cells. As African green monkey BRCA2 is 94% identical (61 of 65 aa) with human BRCA2 in the 2C9 epitope region (aa 1264 to 1329) and 90% identical (304 of 338 aa) with human BRCA2 in the BAC1 region (aa 829 to 1162), this most likely represents detection of endogenous Cos BRCA2. No bands at this molecular weight were detected using control mAb P3x63 or normal rabbit serum.

Figure 4: MAb 2C9 immunoprecipitates a high molecular weight protein consistent with BRCA2 from breast and ovarian carcinoma cells. MCF-7 cells (panels A and B) were serum starved for 3 days, then released from starvation for 1 day, followed by lysis and protein extraction. The lysate from 17 x 106 cells (panel A) or 56 x 106 cells (panel B) was immunoprecipitated with mAb 2C9 as described. Immunoprecipitates were separated on a 4% SDS polyacrylamide gel, and Western blot analysis was performed. Bands were detected with either polyclonal anti-BAC2 serum (panel A) or with 2C9 (panel B). In panel C, protein lysates from 61 x $10^6$ T47D cells (lanes 1-4), 54 x $10^6$ BT483 cells (lanes 9-12), 52 x $10^6$ MCF-7 cells (lanes 13-16), and 60 x $10^6$ SKOV3 cells (lanes 5-8) were immunoprecipitated with mAb 2C9, followed by separation on a 5% non-denaturing polyacrylamide gel, Western blot analysis, and detection with 2C9. A band consistent with the 384 kDa predicted MW of BRCA2 protein is specifically immunoprecipitated by 2C9 in all cases (lanes 3, 7, 11, 15). No specific reactivity is seen with proteins immunoprecipitated by negative control antibody (preclear #1-lanes 1, 5, 9, 13; preclear #2-lanes 2, 6, 10, 14), or with proteins remaining in the lysate following 2C9 immunoprecipitation (lanes 4, 8, 12, 16). In panels B and C, lower molecular weight bands likely correspond to products of mRNA splice variants, differentially processed protein, or protein degradation products. In panel A, the 220 kDa molecular weight marker was run off the gel; lower molecular weight bands may have been run off as well.

Figure 5: BRCA2 mAb 2C9 is reactive with terminally differentiated epithelial cells in thymus. In a comprehensive immunohistochemical screen of frozen acetone-fixed tissue sections to identify tissues with potentially higher levels of BRCA2 protein, we found strong cytoplasmic reactivity with 2C9 mAb in the terminally differentiating thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (panel B). Panel A shows reactivity with isotype-matched control mAb, P3X63. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

Figure 6: BRCA2 mAb 2C9 is reactive with terminally differentiated cells in adult human skin. MAb 2C9 reacts with the suprabasal layers of adult epidermis (panel B) and with eccrine ducts in the dermis (panel E). Panels A and D show corresponding negative reactivity with isotype-matched control mAb P3X63. For comparison, the pattern of reactivity with mAb AE-2 (panels C, F), specific for the 65-67 kDa and 56.5 kDa cytokeratins (CK 1, 2, and 10) characteristic of terminally differentiated epithelial cells (Moll 1982, Woodcock-Mitchell 1982), differs from that seen with mAb 2C9. MAb 2C9 reacts with eccrine ducts but not with the secretory portion of the eccrine coil (panel E), while AE-2 demonstrates significant reactivity with all portions of the eccrine gland (panel F). In addition, while 2C9 shows uniform reactivity with suprabasal layers of the epidermis (panel B), AE-2 shows granular staining, with increasing reactivity with distance above the basal epidermal layer (panel C). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

Figure 7: BRCA2 mAb 2C9 reacts with squamous cell carcinomas. MAb 2C9 demonstrated strong reactivity with 9/10 of well differentiated squamous cell carcinomas (SCCs) of head or neck origin. No reactivity was seen with isotype-matched negative control mAb P3X63 (not shown). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

Figure 8: BRCA2 mAb 2C9 reactivity in cultured epidermal keratinocytes. 2C9 reactivity was localized using immunoperoxidase assays on epidermal keratinocytes derived from neonatal foreskins and cultured in situ onto glass slides. 2C9 immunoreactivity was cytoplasmic and in many cells, appeared to be perinuclear, with a thin rim of unstained cytoplasm present between the nucleus and the greatest intensity of staining. Nuclear dots were notable at high magnification (panel B, arrow). Reactivity was strongest on larger, more squamous cells. Similar results were obtained using immunofluorescence staining. The overall staining intensity of cultured keratinocytes is much lower than that seen in tissue sections of epidermis, suggesting that factors which up-regulate expression of BRCA2 protein in vivo are not present in our keratinocyte cultures. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

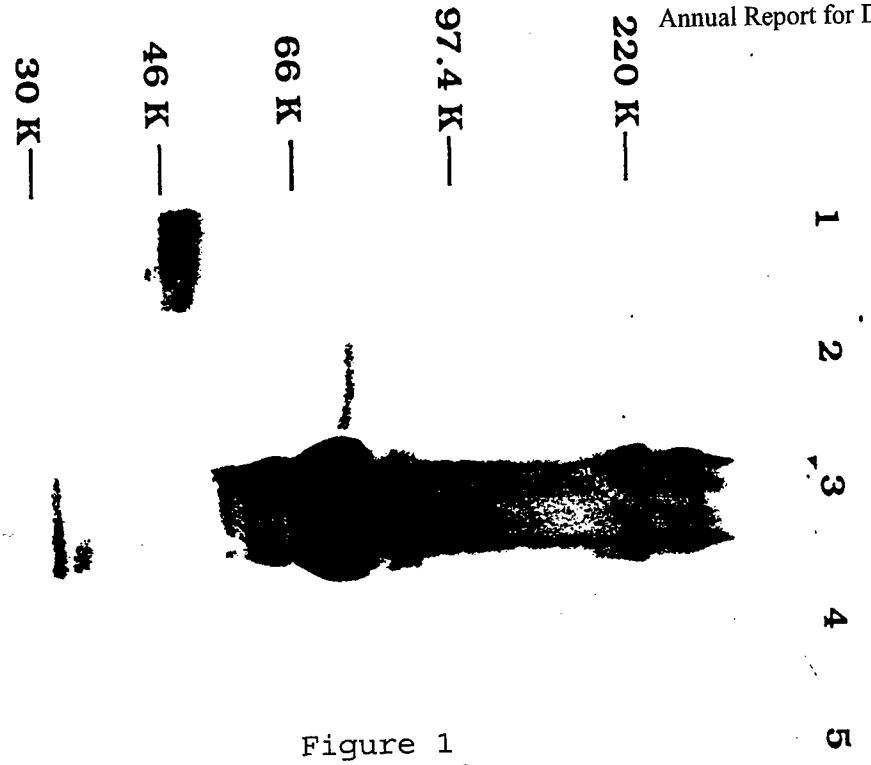


Figure 1

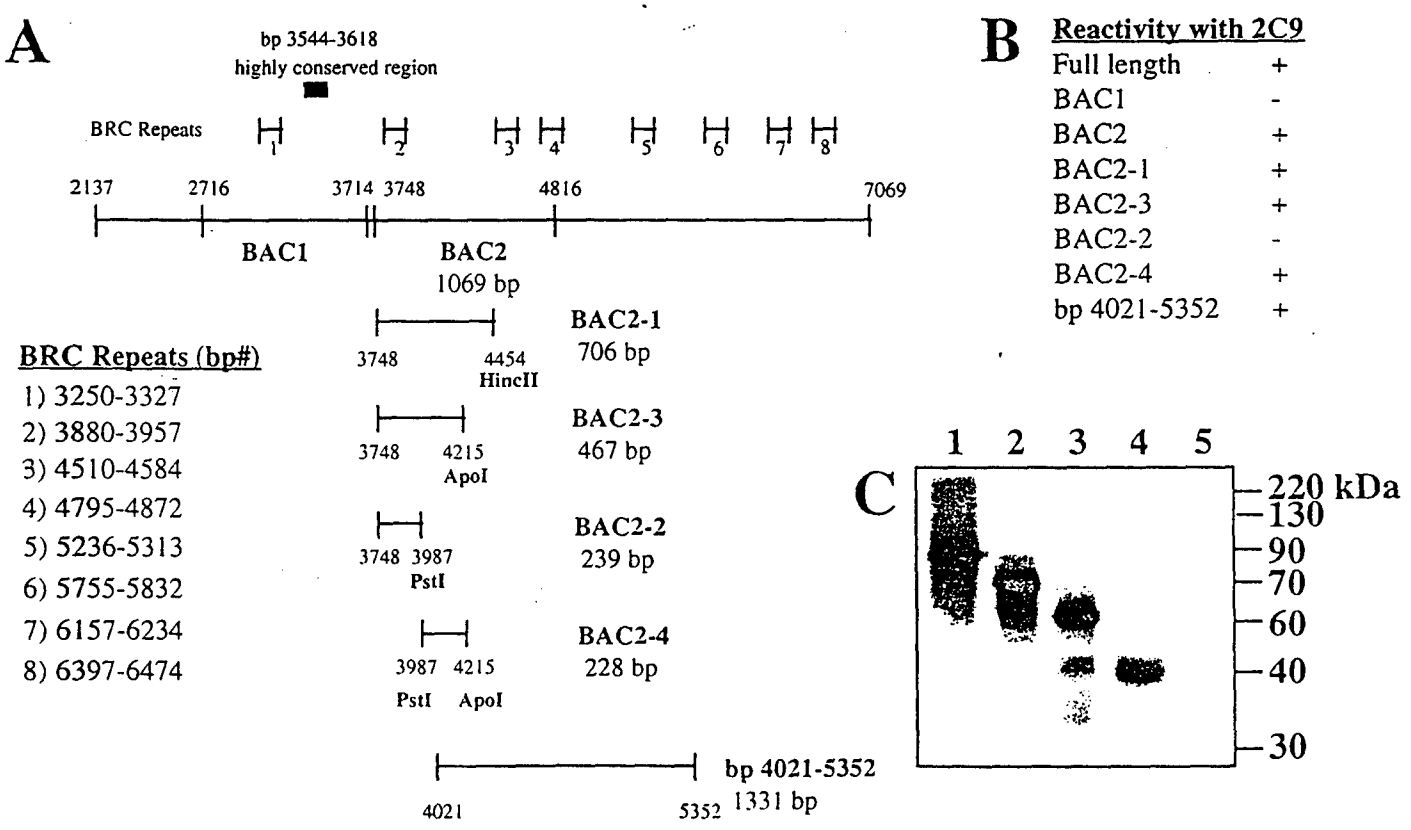


Figure 2

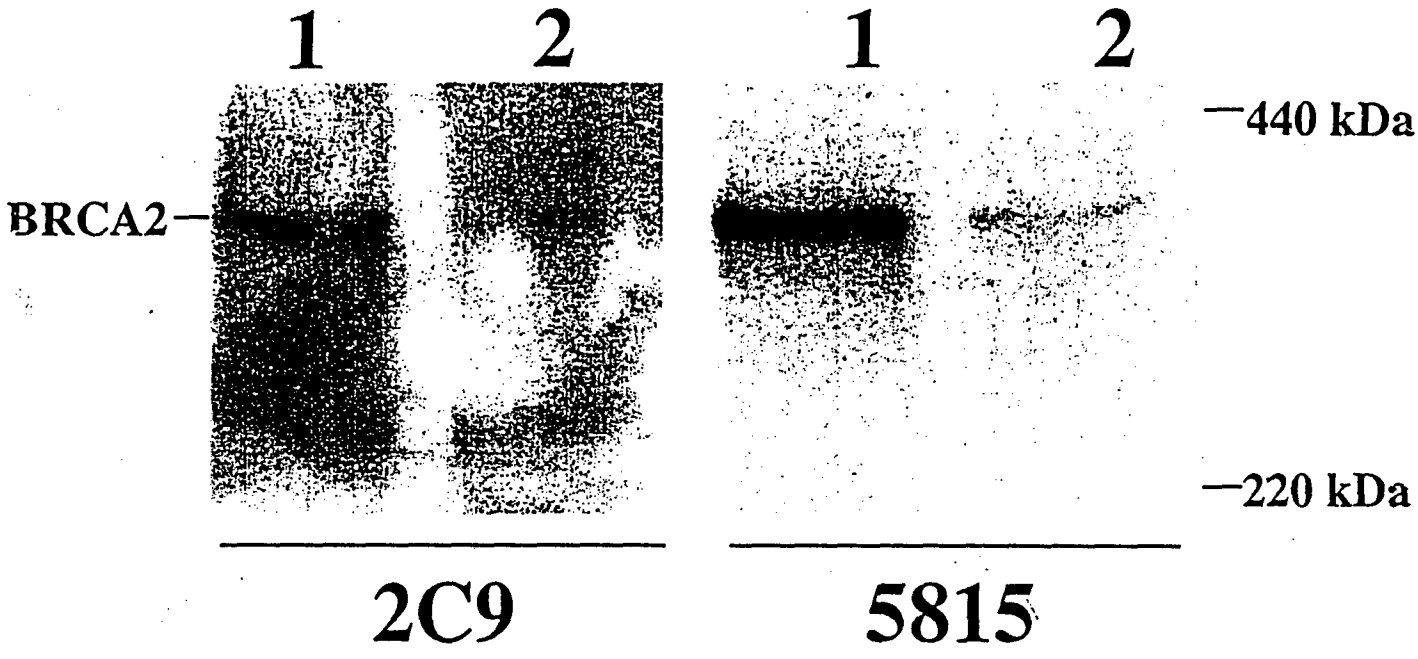


Figure 3

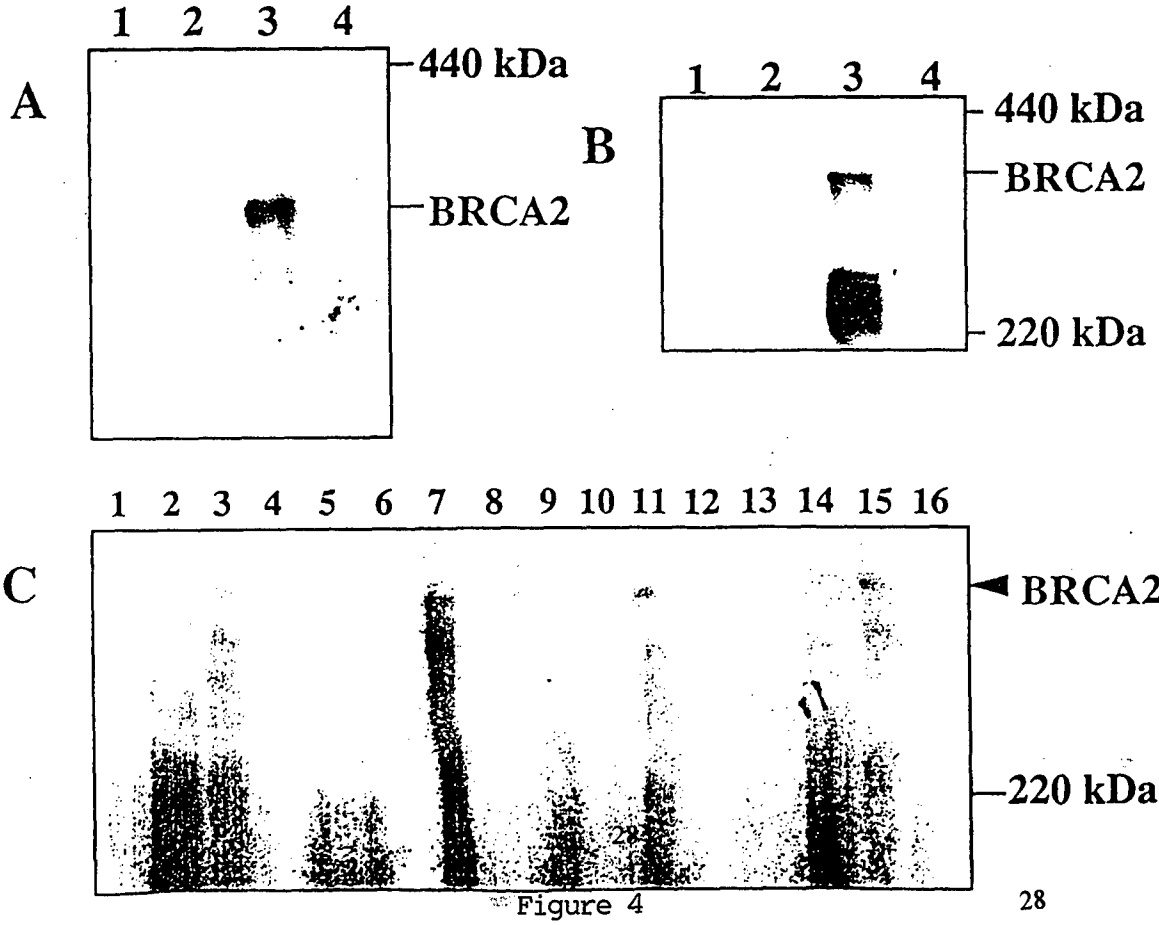


Figure 4

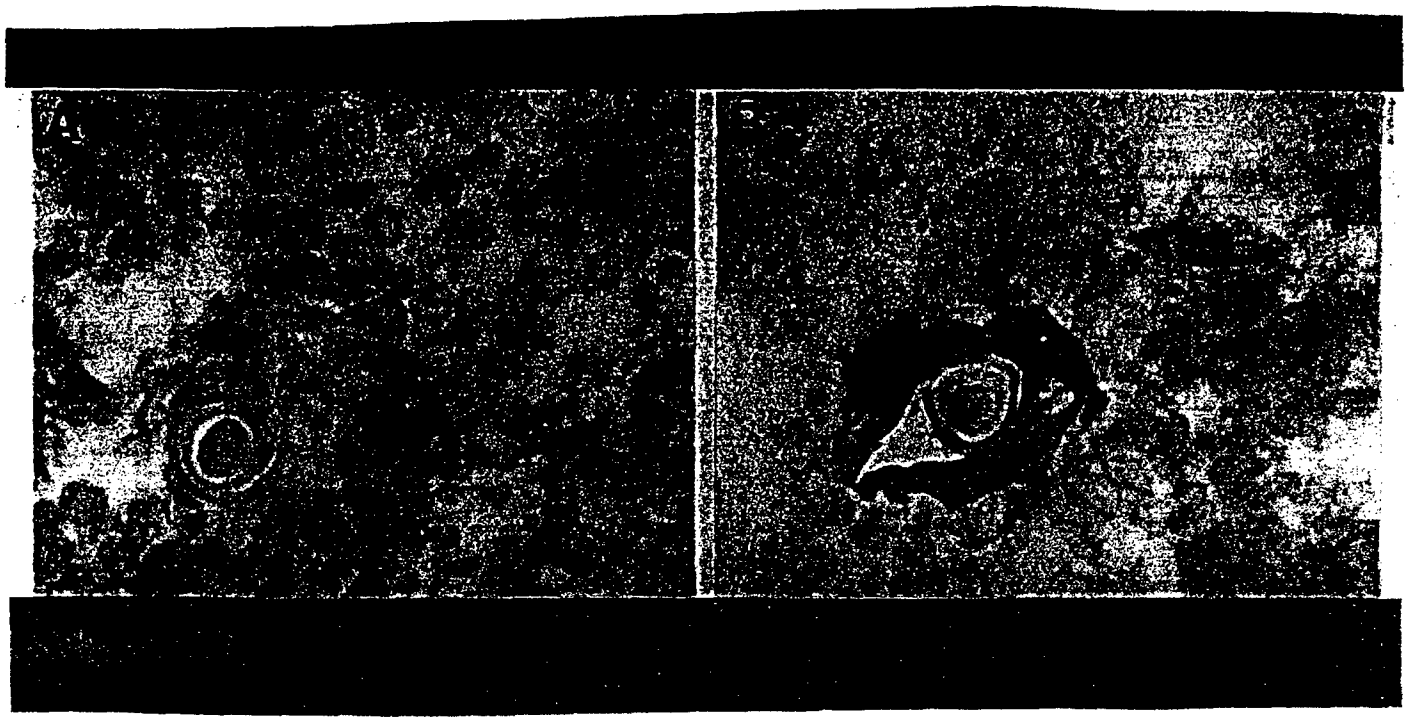

Figure 5

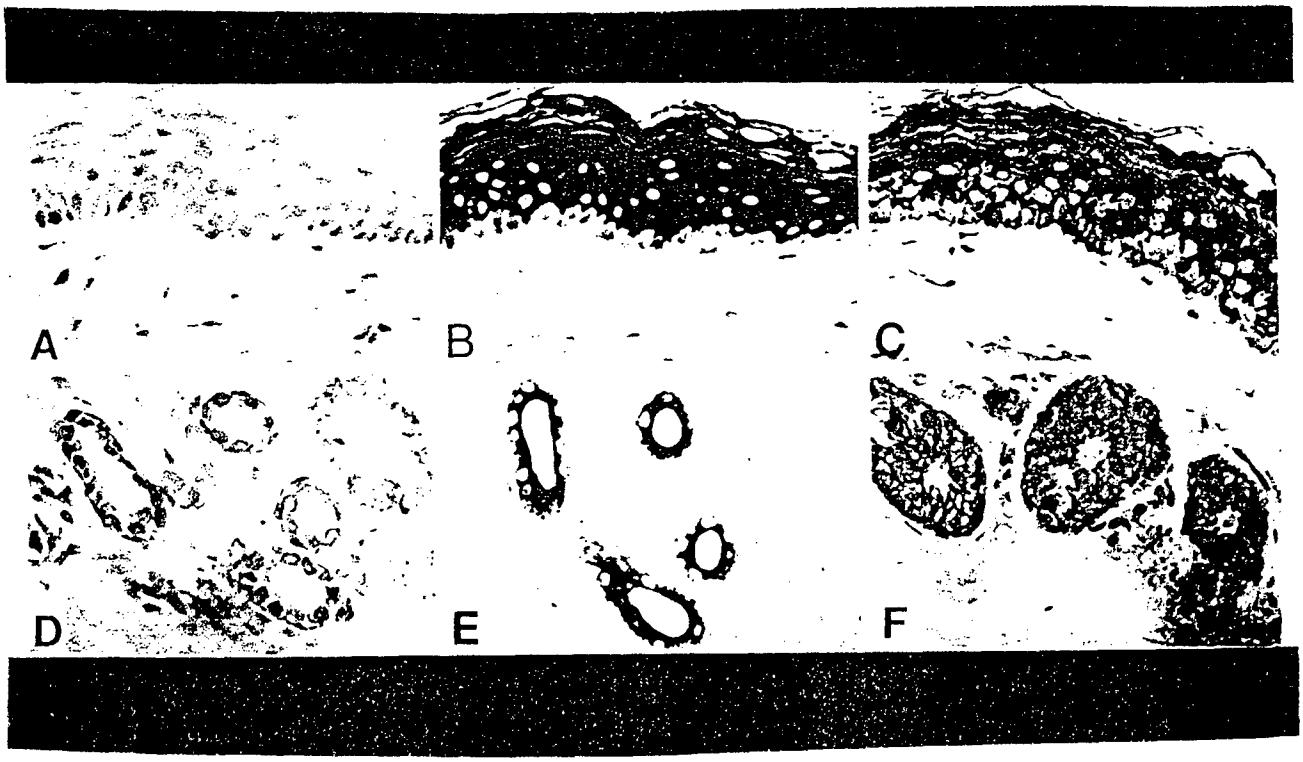


Figure 6

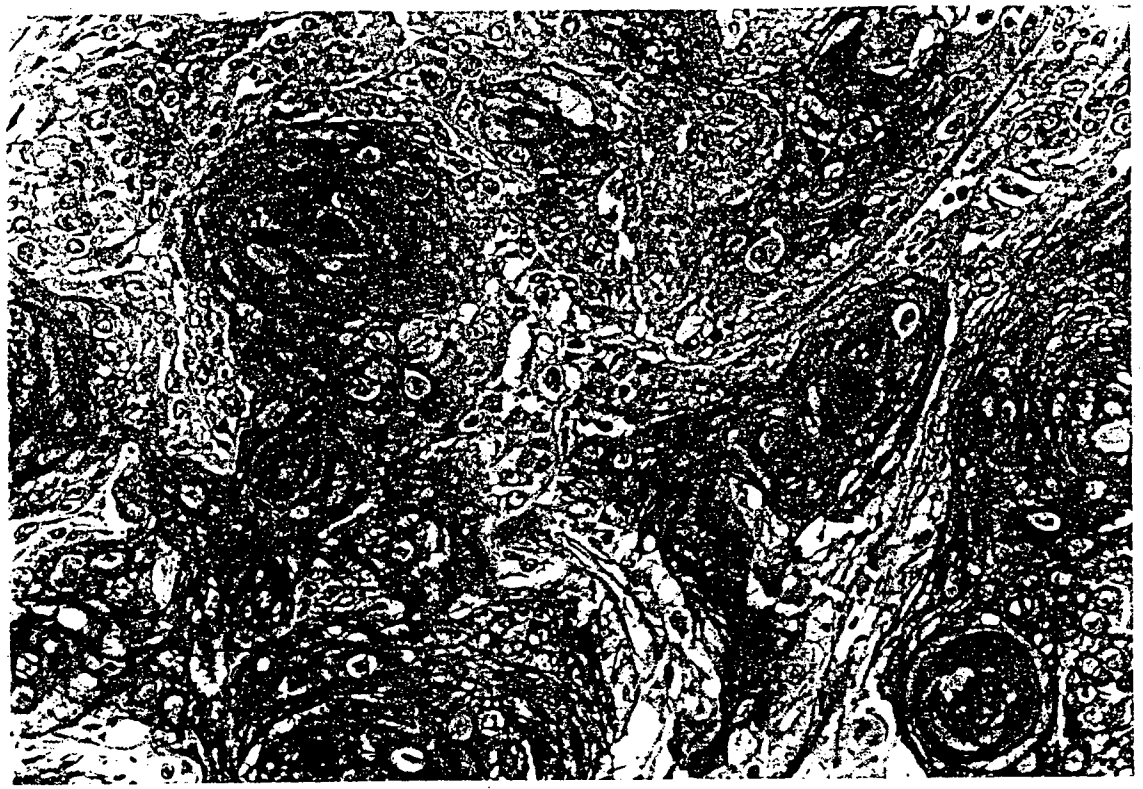

Figure 7

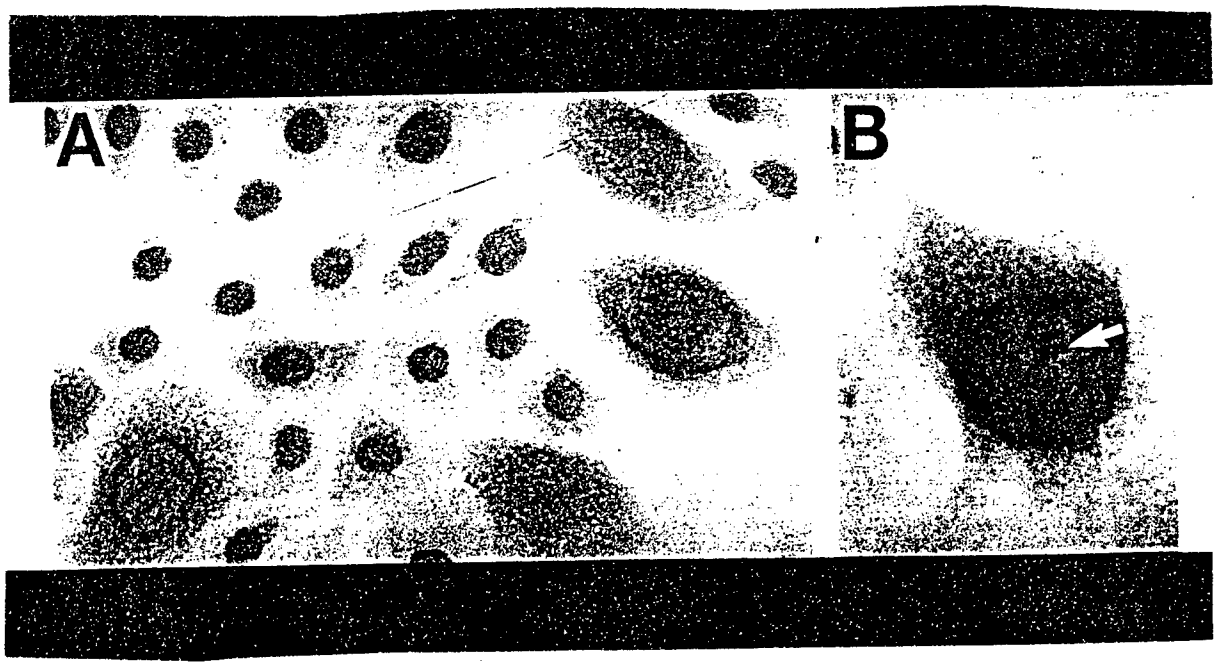


Figure 8

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

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